AD_____

Award Number: W81XWH-10-1-0014

TITLE: tRNAs as Therapeutic Agents of Breast Cancer

PRINCIPAL INVESTIGATOR: Tao Pan, Ph.D.
Marsha Rosner, Ph.D.

CONTRACTING ORGANIZATION: The University of Chicago
Chicago, IL 60637

REPORT DATE: July 2013

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
July 2013	Final Addendum	1 July 2012 - 30 June 2013

4. TITLE AND SUBTITLE

tRNAs as Therapeutic Agents of Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0014

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Tao Pan, Marsha Rosner
Donghui Zhou, Renaud Geslain, Qing Dai, Jiyong Lee, Casey Frankenberger, Eva Eves

E-Mail: taopan@uchicago.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Chicago
Chicago, IL 60637

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Breast cancer refers to a variety of types that each derive from distinct causes and merit different treatments. It is critical to apply a variety of treatment options that target a wide range of cellular mechanisms and targets, including those that are previously unexplored to enable new routes of therapy. Our proposal explores a new and previously under-appreciated avenue which is to use specifically engineered tRNAs that are always attached with an incorrect amino acid in a human cell. This mismatch of amino acid identity to the coding capacity of the tRNA results in increased production of mutated proteins which are misfolded, leading to over-simulation of an intrinsic cellular process that causes cell death. We are studying the effect of these engineered, mischarged tRNAs on breast cancer cells. Ultimately, we aim to demonstrate the suitability of these engineered RNAs as a new class of agents for the treatment of breast tumors.

15. SUBJECT TERMS

tRNA, therapy, delivery, breast cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Final Report

Proposal : W81XWH-10-0014,-0015, tRNAs as therapeutic agents for Breast Cancer.
Period: 07/1/10-06/30/13.
PIs: Tao Pan, Marsha Rosner.

INTRODUCTION:

Transfer RNAs (tRNA) are small non-coding RNAs that read the genetic codes in protein synthesis. It is essential for the proliferation, fitness and adaptation of the cell that each tRNA is aminoacylated (charged) with its designated amino acid. The utilization of mischarged tRNAs (i.e. tRNAs with incompatibly charged amino acid and decoding capacity) leads to the synthesis of mutated proteins that can fold incorrectly. Accumulation of misfolded proteins in the cell activates an integrated cellular mechanism, the Unfolded Protein Response which dictates cell fate in response to the amount of misfolded proteins. High accumulation of misfolded proteins derived from the cellular presence of mischarged tRNAs can therefore induce apoptosis of the cell. We aim to engineer tRNAs that are always mischarged in a human cell and study their effects on breast tumor cell physiology and cell death. Protein demand in rapidly proliferating cells is extremely high and small defects during cellular protein synthesis caused by the presence of such tRNAs can have a strong impact on both tumor invasiveness and survival. Ultimately, we aim to demonstrate that these mischarged tRNAs can be developed as a novel class of RNA-based agents to treat breast tumors.

BODY:

The figures cited in Tasks 1 and 2 are in the appended publication: Zhou et al.: Anti-tumor effects of an engineered "killer" transfer RNA, *Biochem. Biophys. Res. Comm.* 427, 148-153 (2012).

Task 1: Confirm that mischarged-tRNA (termed “killer-tRNA” for its ability to kill cancer cells) works in two breast cancer cell lines. → **Completed successfully.**

We analyzed the impact on two model strains: MDA-MB231 (Her2-) and BR474 (Her2+). Killer-tRNA blocks overall translation at 24h and kill cells at 48h in a dose dependant fashion as shown by lipofection of different amounts of killer-tRNA (Fig. 1). Wild-type $tRNA^{Ser}$ was used as a control. Killer-tRNA also drastically reduces growth rate of both breast cancer cell lines (Fig. 2).

Task 2: Examine the effect of mischarged tRNA (killer-tRNA) in mice xenograft tumor models. → **Completed successfully.**

In order to test the therapeutic potential of killer tRNA, its effect on tumor formation needs to be evaluated in animals. In cell cultures, killer tRNA showed strong inhibitory effects when the transfection period was for at least 24 hours. In therapeutic settings, however, shorter treatment time is desirable. Our goal here is to determine whether shorter treatment with killer tRNA would be sufficient to inhibit xenograft tumor formation in mice. We chose to use MDA-MB-231 derived cells containing a stably integrated luciferase gene (MDA-MB-231+luc) for this experiment. The incorporation of luciferase in these cells enabled us to monitor tumor formation by whole animal imaging. MDA-MB-231 cells are highly metastatic and have been used routinely in the field as a model system for xenograft tumors.

We first optimized the amount of killer tRNA and the treatment time needed prior to mouse injection (Fig. 3). One million cells are needed for each mouse injection. To optimize the killer tRNA level and transfection time, we initially used 6-well plates containing $3x10^5$ cells per well and varied the dosage of killer tRNA over a 24 hour transfection period. We found that ~800 ng per well killer tRNA represented the optimum concentration for transfection. Higher dosage led to greater side effects for even the wild-type tRNA, whereas lower dosage decreased the effect of killer tRNA. We then varied the length of transfection time at 800 ng killer tRNA per well to determine whether killer tRNA treatment can be reduced to less than 24 hours. In this experiment, killer tRNA was transfected for the indicated amount of time, both killer tRNA and the transfection reagent were then removed from the medium, and the cells were incubated for another 24 hours before analysis by the Wst-1 assay. We found that 8 -16 hours killer tRNA treatment provides stable inhibition for MDA-MB-231+luc cells.

These conditions were used for killer tRNA treatment and dosage during the actual mouse injection experiment. To obtain 10^6 cells for injection per mouse, we further scaled up killer tRNA treatment. MDA-MB-231+luc cells in 10 cm plates at 80% confluence were transfected with 4000 ng killer or wild-type tRNA, or no-tRNA but containing the transfection reagent and incubated for 12 hours. The transfection reagent and tRNA were removed, and cells were returned to normal medium for another 12 hours to minimize the undesirable side effects induced by the transfection reagent. These three groups of cells were then harvested for mouse injection. A small aliquot of these same cells was also cultured to confirm that the killer tRNA treatment indeed led to inhibition of cell growth.

Consistent with cell culture results, killer tRNA had a large inhibitory effect on breast tumor formation in a xenograft mouse model (Fig. 4). We injected 10^6 cells into each mouse and monitored tumor formation after 8 and 33 days. As expected, 8/9 mice in the no-tRNA group developed tumors, and 7/10 mice in the wild-type tRNA group developed tumors after 8 days. In contrast, no mice (0/10) in the killer tRNA group developed tumors after 8 days. The same result was obtained at 33 days post-injection. These results demonstrate that killer tRNA exhibits a strong inhibitory effect on tumor formation, and twelve hours of treatment are sufficient to achieve complete inhibition.

Task 3: Explore delivery methods of killer-tRNA to mouse tumors. → **Completed.**

a. *Design tRNA scaffolds that enable the expression of the conditional reporter.* → *Completed successfully.*

In comparison with standard lipofection, the efficiency of nanoparticle-mediated delivery is expected to be in the low to mid range. The onset of the apoptotic response associated with killer tRNA is dose dependant. Therefore, rather than waiting for complex phenotypical changes in the targeted cells we proposed to design a fluorescent screening procedure to monitor the internalization and the usage in translation of tRNA based drugs. This procedure includes a set of three similar molecules: killer-tRNA, wild-type tRNA, and suppressor tRNA (Fig. 5). They share identical $tRNA^{Ser}$ bodies (96 % of the molecule) but display different anticodons. Suppressor tRNA was engineered to base pair with amber stop codons and support translation of reporter proteins harboring amber non-sense mutations in their open reading frame. Delivery strategies effective for the suppressor tRNA will be directly applicable to the killer-tRNA, its toxic mimic.

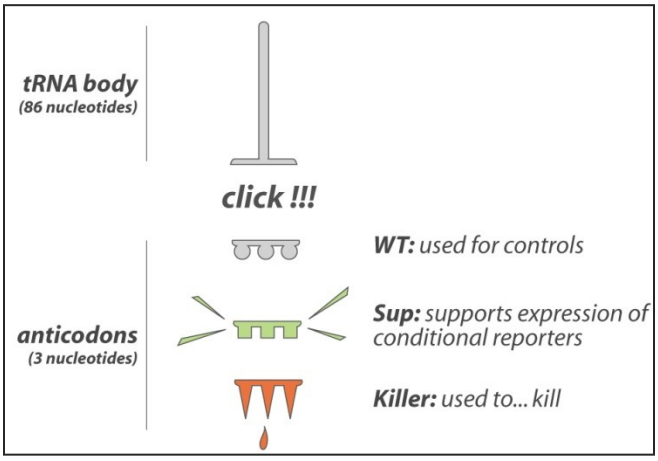

Figure 5. Scaffold of three tRNAs used in the delivery study. These tRNA differ only in their anticodon sequence. Wild-type tRNA reads codon for serine, Suppressor (Sup) tRNA for amber stop, and killer tRNA for isoleucine.

b. Design and test of a tandem meGFP (constitutive) and mCherry (conditional) reporter. → Completed successfully.

These two tandem protein reporters have distinct excitation and emission spectrums allowing simultaneous study by flow cytometry and fluorescent microscopy. The gene encoding the fusion protein harbors a non-sense mutation in the linker between meGFP (green) and mCherry (red). In the absence of suppressor tRNA, only meGFP could be expressed (tested here with Hela cells for transfection convenience, Fig. 6). Co-transfection of suppressor tRNA allowed the read-through of the non-sense mutation and supported the expression of the full-length dual reporter. We thus established the basis for a simple screening procedure to monitor tRNA incorporation into cells based on fluorescence.

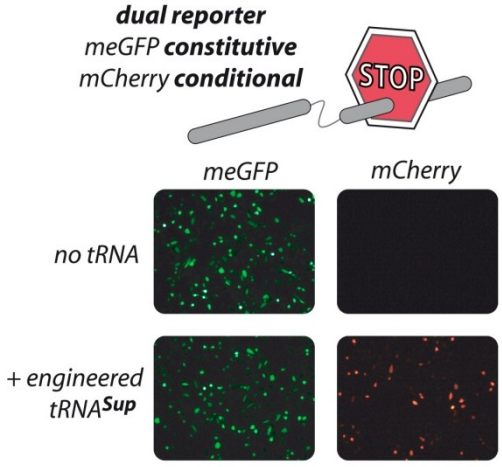

Figure 6. Design and test of a dual reporter to monitor delivery efficiency. The meGFP signal indicates cells that contain this dual reporter, whereas mCherry signal is only present when cells are successfully transfected with tRNA.

c. Nanoparticle delivery study. → Discontinued due to technical difficulties.

We attempted to deliver our set of three tRNAs using nanoparticles formulated by LNK Chemsolutions (Lincoln, NE). We found that the nanoparticles in our study are able to deliver a small organic fluorescent molecule in cultured cells. Unfortunately, we found that these same nanoparticles cannot efficiently package tRNA, likely due to the highly charged nature of tRNA molecules. Other nanoparticle formulas will be needed.

d. Increase killer tRNA effectiveness in tumor cells. → Completed.

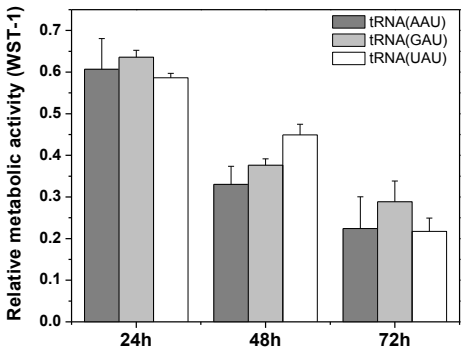

Figure 7. Toxicity test of killer tRNA variants with distinct Ile-anticodons. The original killer tRNA has the anticodon of AAU. tRNAs are transfected into MDA-MB231 cells and the metabolic activity (a proxy of cell death) measured after 24-72h.

We searched for killer tRNA variants that may be applied in lower doses for breast tumor treatments. These variant killer tRNAs contain distinct anticodons that may increase their efficiency in translation thereby increasing ER stress. Among the three variants tested (Fig. 7), all show similar killing effects as the original killer tRNA (AAU) for breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated that killer tRNA is effective for a wide range of cancer cells.
- Constructed dual reporter system for efficient monitoring of tRNA delivery.
- Determined optimal conditions for killer tRNA study in animals.
- Demonstrated that killer tRNA strongly inhibits mouse xenograft tumor formation.

REPORTABLE OUTCOMES:

- A manuscript describing the results under Tasks 1 and 2 has been published in *Biochem. Biophys. Comm (BBRC), 427, 148-153 (2012)*. Title: Anti-tumor effects of an engineered "killer" transfer RNA. Authors: Dong-hui Zhou, Jiyoung Lee, Casey Frankenberger, Renaud Geslain, Marsha Rosner, Tao Pan.
- Constructs of killer, suppressor and wild-type tRNAs.
- Construct of dual reporter plasmid.

CONCLUSION:

Our results show for the first time that killer tRNAs are effective agents for eliminating breast cancer cells. We show that killer tRNA very effectively alters cellular protein synthesis and leads to cell death in breast cancer cells. We also show that a short time treatment of killer tRNA eliminates tumor growth in mice xenograft tumor models. We found so far that our original killer tRNA has the highest killing effect among several variants tested. Future work will include finding more potent killer tRNA variants which would allow lower doses of killer tRNA to be used, thus alleviating the necessity of developing stringent delivery methods. We also need better delivery methods to enable efficient delivery of killer tRNAs into breast cancer tumors.

RNA interference has become an important new class of drug and considerable efforts have been made to develop methods to shuttle small interfering RNA (siRNA) molecules into target cells. The primary mode of action of siRNA based approaches is to target and degrade specific messenger RNAs. Our tRNA-based therapeutic approach is based on an entirely different

mechanism utilizing a completely different pathway compared to siRNA therapeutics. We envision that our approach will complement siRNA approaches and offer a different route of treatment for breast cancer.

Bioliography of all publications and meeting abstracts:

Publication:

Zhou, D-H, J-Y. Lee, C. Frankenberger, R. Geslain*, M. Rosner*, **Tao Pan***: Anti-tumor effects of an engineered "killer" transfer RNA, *Biochem. Biophys. Res. Comm.* 427, 148-153 (2012).

Meeting abstracts:

Era of Hope 2011: tRNA as therapeutic agent of breast cancer
Renaud Geslain, Andreas Czech, Eva Eves, Marsha Rosner, Tao Pan

List of personnel receiving pay from the research effort:

1. Tao Pan (PI).
2. Marsha Rosner (PI).
3. Renaud Geslain (Research Associate, worked on Tasks 1, 2 and 3).
4. Dong-hui Zhou (Research Associate, worked on Tasks 2 and 3).
5. Qing Dai (Research Scientist, worked on Task 3).
6. Jiyoung Lee (Postdoctoral Scholar, worked on Tasks 1, 2).
7. Casey Frankenberger (Postodoctoral Scholar, worked on Task 2).
8. Eva Eves, (Senior Research Associate, worked on Tasks 1, 2).

Biochemical and Biophysical Research Communications 427 (2012) 148–153

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Anti-tumor effects of an engineered "killer" transfer RNA

Dong-hui Zhou [a], Jiyoung Lee [b], Casey Frankenberger [b], Renaud Geslain [a,c,*], Marsha Rosner [b,*], Tao Pan [a,*]

[a] Department of Biochemistry & Molecular Biology, University of Chicago, Chicago, IL 60637, USA
[b] Ben May Department for Cancer Research, University of Chicago, Chicago, IL 60637, USA
[c] Department of Biology, DePaul University, Chicago, IL 60614, USA

ARTICLE INFO

Article history:
Received 5 September 2012
Available online 16 September 2012

Keywords:
Breast cancer
tRNA
Translation
Tumor formation

ABSTRACT

A hallmark of cancer cells is their ability to continuously divide; and rapid proliferation requires increased protein translation. Elevating levels of misfolded proteins can elicit growth arrest due to ER stress and decreased global translation. Failure to correct prolonged ER stress eventually results in cell death via apoptosis. tRNA^{Ser}(AAU) is an engineered human tRNA^{Ser} with an anticodon coding for isoleucine. Here we test the possibility that tRNA^{Ser}(AAU) can be an effective killing agent of breast cancer cells and can effectively inhibit tumor-formation in mice. We found that tRNA^{Ser}(AAU) exert strong effects on breast cancer translation activity, cell viability, and tumor formation. Translation is strongly inhibited by tRNA^{Ser}(AAU) in both tumorigenic and non-tumorigenic cells. tRNA^{Ser}(AAU) significantly decreased the number of viable cells over time. A short time treatment with tRNA^{Ser}(AAU) was sufficient to eliminate breast tumor formation in a xenograft mouse model. Our results indicate that tRNA^{Ser}(AAU) can inhibit breast cancer metabolism, growth and tumor formation. This RNA has strong anti-cancer effects and presents an opportunity for its development into an anti-tumor agent. Because tRNA^{Ser}(AAU) corrupts the protein synthesis mechanism that is an integral component of the cell, it would be extremely difficult for tumor cells to evolve and develop resistance against this anti-tumor agent.

1. Introduction

The ability of cancer cells to develop resistance to drugs remains a huge challenge in the clinical treatment of cancer. A typical drug targets a single or handful of protein or nucleic acid molecules. Drug-resistance occurs through multiple mechanisms. Often, as in the case of the EGF receptor or the BCR-Abl kinase, target mutations eliminate or reduce specific drug-target interactions [1,2]. Alternatively, drug-resistance can result from induced expression of pumps that reduce the intracellular drug concentration [3,4]. Resistance can also arise by constitutive activation of an alternate pathway that bypasses the original drug target. For example, Raf inhibitors effectively suppress melanoma driven by the V600E-BRaf mutation but subsequent activating Ras mutations enable stimulation of the MAP kinase pathway via Raf-1 [5].

Our aim is to develop a potential therapeutic agent that has the potential to significantly reduce the probability that cancer cells will develop resistance to it. This agent should have a very broad target range, rather than targeting just one or a handful of molecules in cells. This agent should also be a close mimic of cellular components so that it cannot be easily marked by cellular machineries as foreign. We describe here the proof-of-principle application of a specific, engineered transfer RNA (tRNA) that can fulfill both criteria.

The endoplasmic reticulum (ER) is a eukaryotic organelle that performs the major functions of synthesizing and packaging proteins. Overloading of misfolded proteins within ER induces the expression of ER-resident chaperones that facilitate protein folding [6–8]. A pro-apoptotic pathway is triggered when cellular adaptive responses cannot compensate for the protein misfolding-induced ER stress [9]. Hence, prolonged exposure to large amounts of misfolded proteins can lead to apoptosis of cancer cells. One way to constitutively generate large amounts of misfolded proteins is to introduce an engineered tRNA in cells that is aminoacylated with serine but reads codons of a different amino acid during translation [10].

Aminoacyl tRNA synthetase catalyzes the attachment of an amino acid to its cognate tRNA. The human seryl-tRNA synthetase (SerRS) aminoacylates tRNA^{Ser} with serine and is particularly useful for the strategy of applying an engineered tRNA to corrupt the cellular proteome. SerRS does not recognize the anticodon of tRNA^{Ser}, so that modifications of the anticodon of tRNA^{Ser} will

Abbreviations: tRNA^{Ser}(AAU), an engineered tRNA derived from human tRNA^{Ser}(AGA). The anticodon sequence is changed from AGA to AAU. This engineered tRNA is aminoacylated with serine but reads codons for isoleucine; tRNA^{Ser}(AGA), the wild-type, human tRNA^{Ser} with the anticodon sequence of AGA.

* Corresponding authors. Present address: Department of Biology, DePaul University, Chicago, IL 60614, USA (R. Geslain).

E-mail addresses: rgeslain@depaul.edu (R. Geslain), m-rosner@uchicago.edu (M. Rosner), taopan@uchicago.edu (T. Pan).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.09.028

not interfere with the ability of this tRNA to be fully aminoacylated [11]. Replacement of serine with other anticodons generates chimeric tRNAs. These chimeric tRNAs are charged with serine but read codons for other amino acids, thus producing large amounts of mutant proteins. Among many non-serine anticodons tested in HeLa, $tRNA^{Ser}$ with the AAU anticodon ($tRNA^{Ser}$(AAU)) leads to the substitution of isoleucine with serine within the proteome and is particularly pro-apoptotic [10].

In this work, we investigate the potential of $tRNA^{Ser}$(AAU) RNA as a therapeutic agent for breast cancer. Although previous work with HeLa cells using transfected plasmids containing $tRNA^{Ser}$(AAU) established that this particular chimeric tRNA is a potent inducer of cell apoptosis [10], it was not known whether short treatment of breast cancer cells with the purified tRNA would be sufficient to kill cells in culture and alter tumor-forming potential in animals. Both aspects are important for therapeutic purposes: therapy will be much simpler when using the tRNA molecule directly instead of plasmid DNA, which typically has a 24–48 h half-life in cells [12]. Furthermore, treatment will be potentially less toxic to normal cells if it lasts only hours instead of days. Here we show that $tRNA^{Ser}$(AAU) efficiently inhibits cancer cell viability in culture and tumor formation in mice, indicating that $tRNA^{Ser}$(AAU) RNA indeed has potential as an anti-cancer drug.

2. Materials and methods

2.1. Preparation of the $tRNA^{Ser}$(AAU) and $tRNA^{Ser}$(AGA)

Plasmid vectors containing the gene for $tRNA^{Ser}$(AAU) and $tRNA^{Ser}$(AGA) downstream to a T7 RNA polymerase promoter were used as templates for run-off transcription [13]. DNA templates were first subjected to 3 h digestion by BstNI at 60 °C, followed by phenol/chloroform extraction and ethanol precipitation. Transcription of these tRNAs was carried out at 37 °C with 2 mM each ATP, GTP, CTP, UTP, and 4.8 mM 5′GMP for 4 h. tRNA was purified on 10% denaturating PAGE and extracted by soaking gel slices overnight at 4 °C in 50 mM KOAc, 200 mM KCl, pH 7, precipitated and resuspended in H_2O.

The sequences of both tRNAs are: $tRNA^{Ser}$(AAU): 5′GUAGUCGUGGCCGAGUGGUUAAGGCGAUGGACUAAUAAUCCAUUGGGGUCUCCCCCGCGCAGGUUCGAAUCCUGCCGACUACG; and $tRNA^{Ser}$(AGA): 5′GUAGUCGUGGCCGAGUGGUUAAGGCGAUGGACUAGAAAUCCAUUGGGGUCUCCCCCGCGCAGGUUCGAAUCCUGCCGACUACG.

2.2. Cell culture, tRNA transfection and cell assays

MDA-MB-231 cells were grown in DMEM with 10% fetal bovine serum (FBS) at 37 °C, 5% CO_2. BT-474 cells were grown in RMPI-1640 with 10% FBS at 37 °C, 5% CO_2. Three breast epithelial cell lines (MCF10A, 184 A1, 184 B5) were cultured in 1:1 (+I factor, Vectro)-DMEM/F12 (Invitrogen, 11330-032) supplemented with 10% FBS, 5 μg/ml insulin, 10 ng/ml EGF and 0.5 μg/ml hydrocortisone. MDA-MB-231 cells stably expressing thymidine kinase, GFP, and luciferase (MDA-MB-231+luc), were kindly provided by Dr. Andy Minn from the University of Chicago. For transient transfections, cells at 70–80% confluency were transfected with $tRNA^{Ser}$(AAU) or $tRNA^{Ser}$(AGA) transcripts using lipofectamine 2000 (Invitrogen) according to manufecturer's instructions. The amount of RNA used and the length of transfection time are variable parameters and are described in Section 3.

Cells were plated, allowed to grow overnight and transfected with $tRNA^{Ser}$(AAU) and $tRNA^{Ser}$(AGA) with or without GFP plasmid using lipofectamine 2000 the following day. For GFP assay: MDA-MB-231, BT-474, MCF 10A, 184A1 and 184B5 cells were grown in 96-well plates at a density of $1–3 \times 10^4$ cells per well per 100 μl medium. For WST-1 assay, MDA-MB-231 cells were grown in 96-well plates at a density of 10^4 cells per well per 100 μl or 3×10^5 per 2 ml in 6-well plates. For tumor formation assay: MDA-MB-231 cells were grown in 10 cm dishes at $5–6 \times 10^6$ per 10 ml.

To monitor metabolic activity of transfected cells, cells were incubated in 96-well or 6-well plates. Transfection complexes were formed by 1 μl or 5 μl lipofectamine 2000 and $tRNA^{Ser}$(AAU) or $tRNA^{Ser}$(AGA) at room temperature in 50 or 500 μl serum-free Opti-MEM® I Reduced Serum Medium, and drop-wise added to cells in 96-well plates or 6-well plate followed by incubation for 24, 48 or 72 h. WST-1 was added at 1/10th volume of the culture medium. The reaction mixture was incubated for 1 h at 37 °C. The formazan dye produced by metabolically active cells was quantified by a scanning multiwall spectrohoptometer at 440 nm directly in 96-well plates or after aliquot were transfered from the 6-well plate to a 96-well plate.

For GFP assay, transfection complexes were formed by 1 μl lipofectamine 2000, 75 ng GFP and $tRNA^{Ser}$(AAU) or $tRNA^{Ser}$(AGA) at room temperature in 50 μl serum-free Opti-MEM® I Reduced Serum Medium, and drop-wise added to cells in 96-well plates followed by incubation for 24, 48 or 72 h. The GFP signal was measured at Ex485/Em515.

For cell death assays, MDA-MB-231 cells were grown in 96-well plates and incubated with 5 μg/ml of propidium iodide (Sigma Aldrich), and measured by flow cytometry.

2.3. Tumor formation assay

All animal work was done as previously described in Dangi-Garimella et al. [15] and in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Briefly, athymic nude mice (Harlan), 3 groups of 10 each 6–7 weeks of age, were orthotopically injected with cells (10^6) in the lower left fat pad (#4) in 100 μl of PBS. All mice were imaged for luciferase activity at both 8 and 33 days post-injection using the IVIS Spectrum Imaging System (Xenogen). Mice were anaesthetized using 2% isoflurane and injected intraperitoneally with D-luciferin (100 mg/kg in PBS). Images were normalized to the same range of radiance prior to quantification.

In order to get a large amount of cells for mice injection, $tRNA^{Ser}$(AAU) or $tRNA^{Ser}$(AGA) were scaled up from 800 ng to 4000 ng, and lipofectamine 2000 from 5 μl to 30 μl according to the results of cell growth in 6-well plates.

For tumor formation assay, transfection complexes were formed by 30 μl lipofectamine 2000 and 4000 ng $tRNA^{Ser}$(AAU) or $tRNA^{Ser}$(AGA) or no-tRNA at room temperature in 3 ml serum-free Opti-MEM® I Reduced Serum Medium, and drop-wise added to cells in 10-cm dishes. These cells were incubated in this condition for 12 h followed by restoring to normal medium for another 12 h, and then three groups of cells were rinsed twice with 1XPBS and harvested by trypsination. Cell numbers per group were ten times 10^6 cells.

3. Results and discussion

3.1. $tRNA^{Ser}$(AAU) inhibits translation

We first evaluated the effects of $tRNA^{Ser}$(AAU) on translation in tumorigenic breast cancer and non-tumorigenic breast epithelial cells. RNA transcripts of $tRNA^{Ser}$(AAU) and a GFP-encoding plasmid were directly co-transfected into two breast cancer cell lines, MDA-MB-231 and BT-474, as well as three non-tumorigenic epithelial breast cell lines, MCF10A, 184A1 and 184B5. As indicated in previous HeLa studies [10], GFP fluorescence can be used to monitor

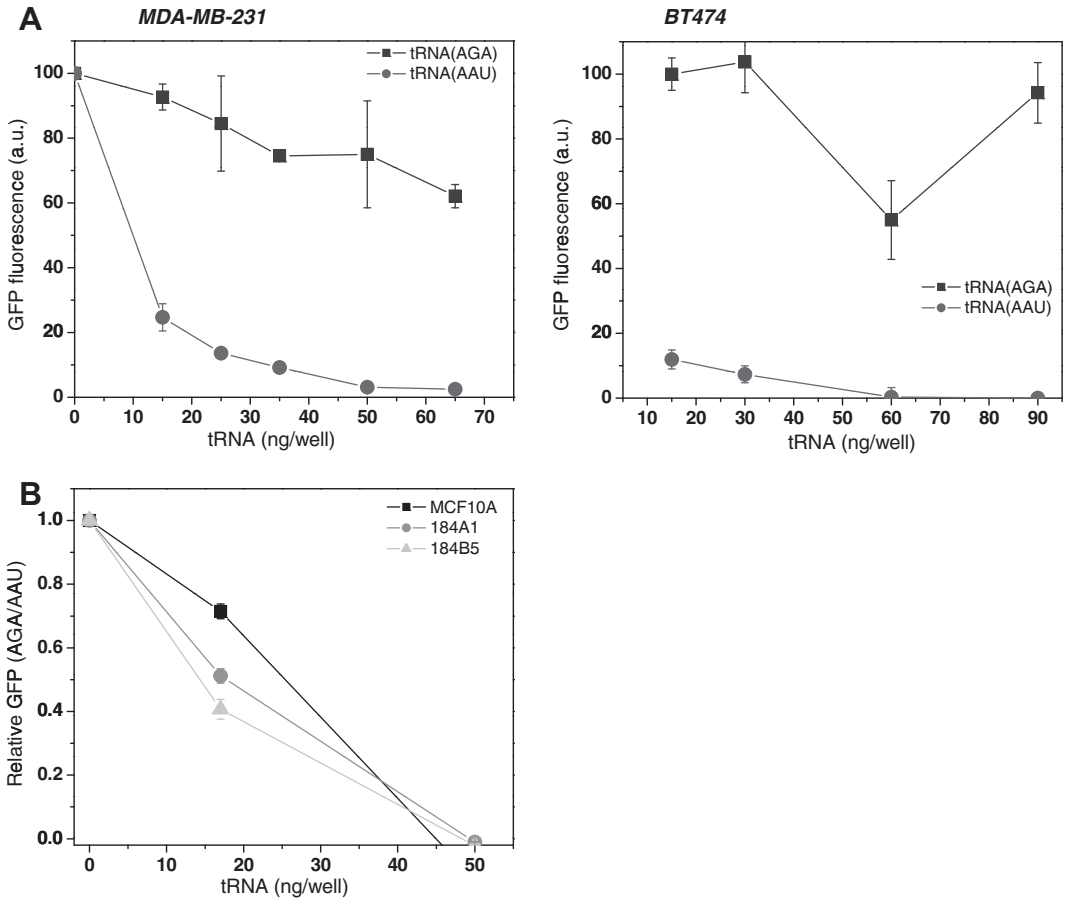

Fig. 1. Synthetic $tRNA^{Ser}$(AAU) transcript inhibits translation in cells. GFP fluorescence was used as a marker of cellular translation activity to measure the impact of $tRNA^{Ser}$(AAU) transfection at different dosages. $tRNA^{Ser}$(AAU) is aminoacylated with serine but reads codons for isoleucine. The control tRNA used here, $tRNA^{Ser}$(AGA) is a wild-type $tRNA^{Ser}$. (A) Two breast cancer cells: MDA-MB-231 and BT-474, 24 h after transfection. Fluorescence was analyzed by flow cytometry and plate reader. (B) Three non-tumorigenic cells: MCF10A, 184A1 and 184B5, 48 h after transfection. Fluorescence was analyzed using a plate reader. Mean values and standard deviations are derived from three independent transfections.

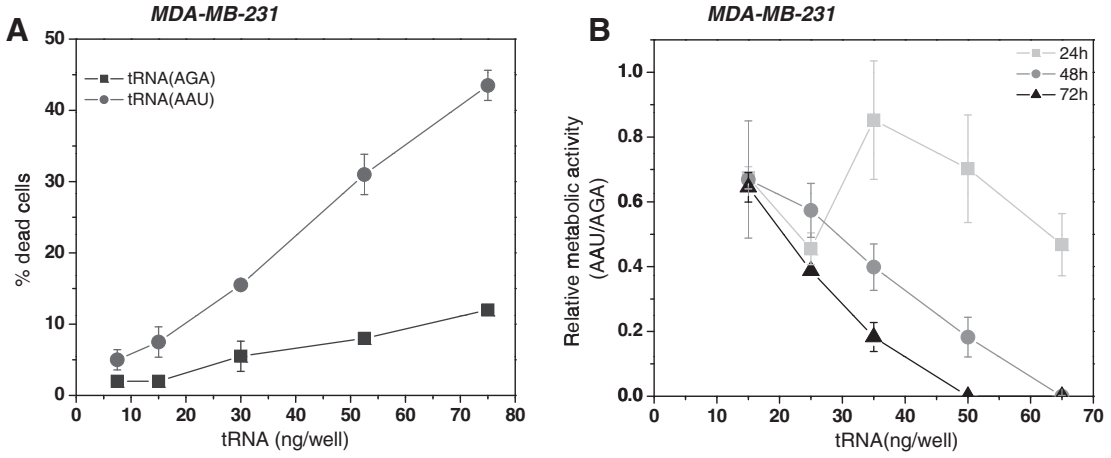

Fig. 2. Synthetic $tRNA^{Ser}$(AAU) transcript increases cell death and inhibits cell growth. (A) Cell death of MDA-MB-231 was evaluated using the dead cell marker propidium iodide in response to different dosage of $tRNA^{Ser}$(AGA) and $tRNA^{Ser}$(AAU), 24 h after transfection. Fluorescence was analyzed by flow cytometry. (B) Effect on cell growth of $tRNA^{Ser}$(AGA) and $tRNA^{Ser}$(AAU), 24, 48 and 72 h after transfection. Cells were monitored at different time using the WST-1 proliferation assay. The data show a reduction in growth rate at a dosage dependent manner. Mean values and standard deviations are derived from three independent transfections.

translation activity of engineered tRNAs and can be easily quantified by flow cytometry or with a plate reader 24 or 48 h after transfection. As a control, the same amount of $tRNA^{Ser}$(AGA) transcript was transfected in parallel. Unlike $tRNA^{Ser}$(AAU) which substitutes Ile-to-Ser in translation, $tRNA^{Ser}$(AGA) is a wild-type $tRNA^{Ser}$. $tRNA^{Ser}$(AAU) does not read any of the GFP codons in our

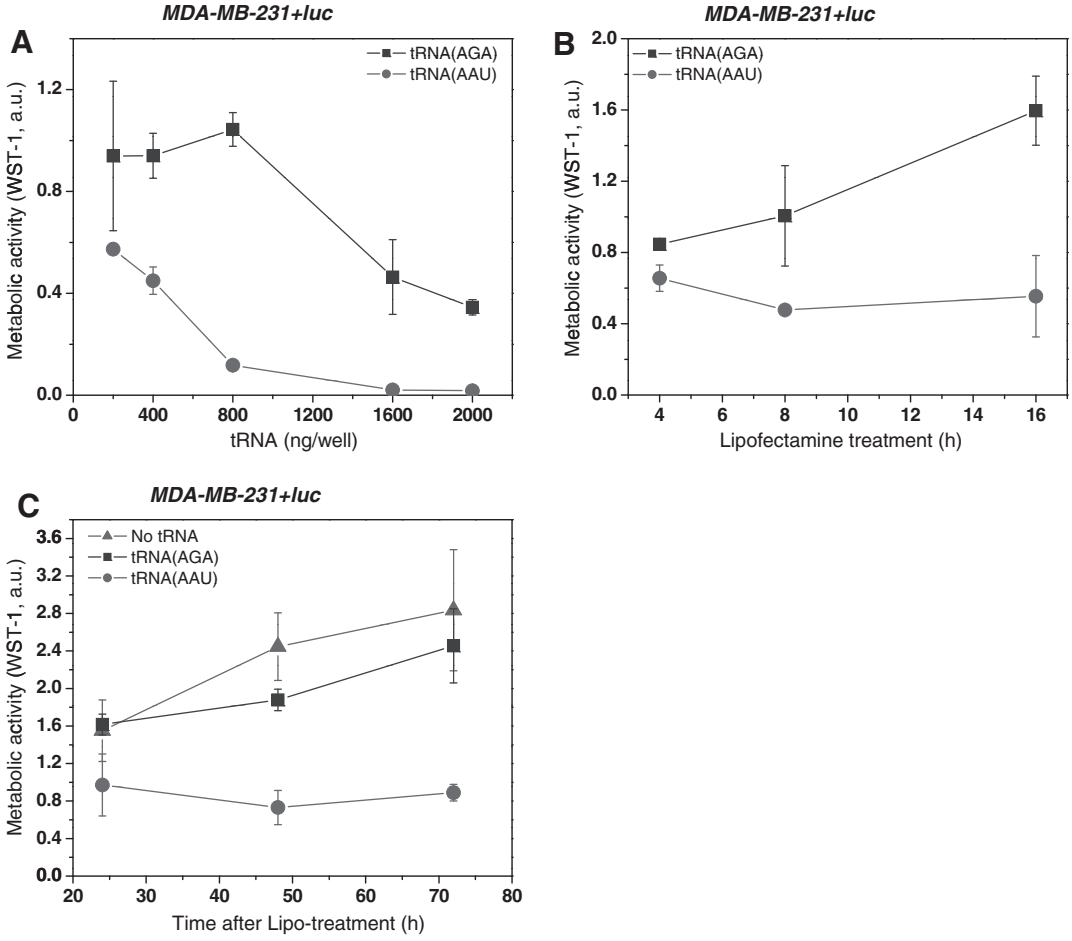

Fig. 3. Optimization of tRNA^{Ser}(AAU) effects for animal experiments. (A) WST-1 proliferation assay was used to monitor the effect of tRNA^{Ser}(AAU) and tRNA^{Ser}(AGA) on MDA-MB-231+luc in 6-well plate 24 h after transfection. (B) MDA-MB-231+luc cells in 6-well plate were first transfected with 800 ng of tRNA^{Ser}(AAU) and tRNA^{Ser}(AGA) per well for the indicated number of hours. tRNA and transfection reagents were then removed and the cells incubated for 24 h in normal medium. (C) WST-1 assay was used to monitor the same three group of cells injected into mice. Cells from no-tRNA, tRNA^{Ser}(AAU)and tRNA^{Ser}(AGA) group were seeded at 30,000 cells per well in a 6-well plate. Growth curves for these three groups at different time are shown. Mean values and standard deviations are derived from three independent transfections.

engineered GFP construct [10]; therefore, its effect on GFP level is not directly related to GFP translation; rather, it reflects global translation of the proteome.

In both tumorigenic cell lines, tRNA^{Ser}(AAU) inhibits translation in a dose dependent manner in 24 h (Fig. 1A). The translation activity was inhibited by 10-fold when ~30–35 ng of tRNA^{Ser}(AAU) were used to transfect 10^4 cells in a 96-well plate. GFP levels dropped to zero when we increased the tRNA^{Ser}(AAU) levels to >60 ng per well. These results demonstrate that tRNA^{Ser}(AAU) is a potent inhibitor of translation in breast cancer cells. tRNA^{Ser}(AAU) dependent inhibition was not selective for cancer cells, however, as all three non-tumorigenic, epithelial cells also exhibited strong inhibition (Fig. 1B). This result indicates that tRNA^{Ser}(AAU) is also toxic for non-tumorigenic cells even though these cells have ~2-fold less cytoplasmic tRNA compared to tumorigenic breast cancer cells [14].

3.2. tRNA^{Ser}(AAU) induces cancer cell death and inhibits cell growth

We then analyzed the effect of tRNA^{Ser}(AAU) on breast cancer cell viability and cell growth. Cell death was quantified using propidium iodide staining and flow cytometry 24 h after transfection (Fig. 2A). For MDA-MB-231 cells, tRNA^{Ser}(AAU) induced high levels of apoptosis, again in a dose dependent manner. At ~60 ng tRNA^{Ser}(AAU) transfected per well, about one third of the cells died within 24 h. At this same tRNA^{Ser}(AAU) concentration, the translation activity was reduced to nearly zero (Fig. 1A). We also monitored the effect of tRNA^{Ser}(AAU) on cell growth using the metabolic indicator, WST-1 (Fig. 2B). tRNA^{Ser}(AAU) similarly reduced the metabolic activity in a dose-dependent manner. At ~60 ng tRNA^{Ser}(AAU) transfected per well, the metabolic activity was reduced to zero after 48 h, although substantial level of activity was still present at 24 h. These results are consistent with tRNA^{Ser}(AAU) acting first to result in translational arrest, followed by the cessation of metabolic activity and cell death.

3.3. tRNA^{Ser}(AAU) inhibits tumor formation in mice

In order to test the therapeutic potential of tRNA^{Ser}(AAU), its effect on tumor formation needs to be evaluated in animals. In cell cultures, tRNA^{Ser}(AAU) showed strong inhibitory effects when the transfection period was for at least 24 h. In therapeutic settings, however, shorter treatment time is desirable. Our goal here is to determine whether shorter treatment with tRNA^{Ser}(AAU) would be sufficient to inhibit xenograft tumor formation in mice. We chose to use MDA-MB-231 derived cells containing a stably integrated luciferase gene (MDA-MB-231+luc) for this experiment. The incorporation of luciferase in these cells enabled us to monitor tumor formation by whole animal imaging [15]. MDA-MB-231

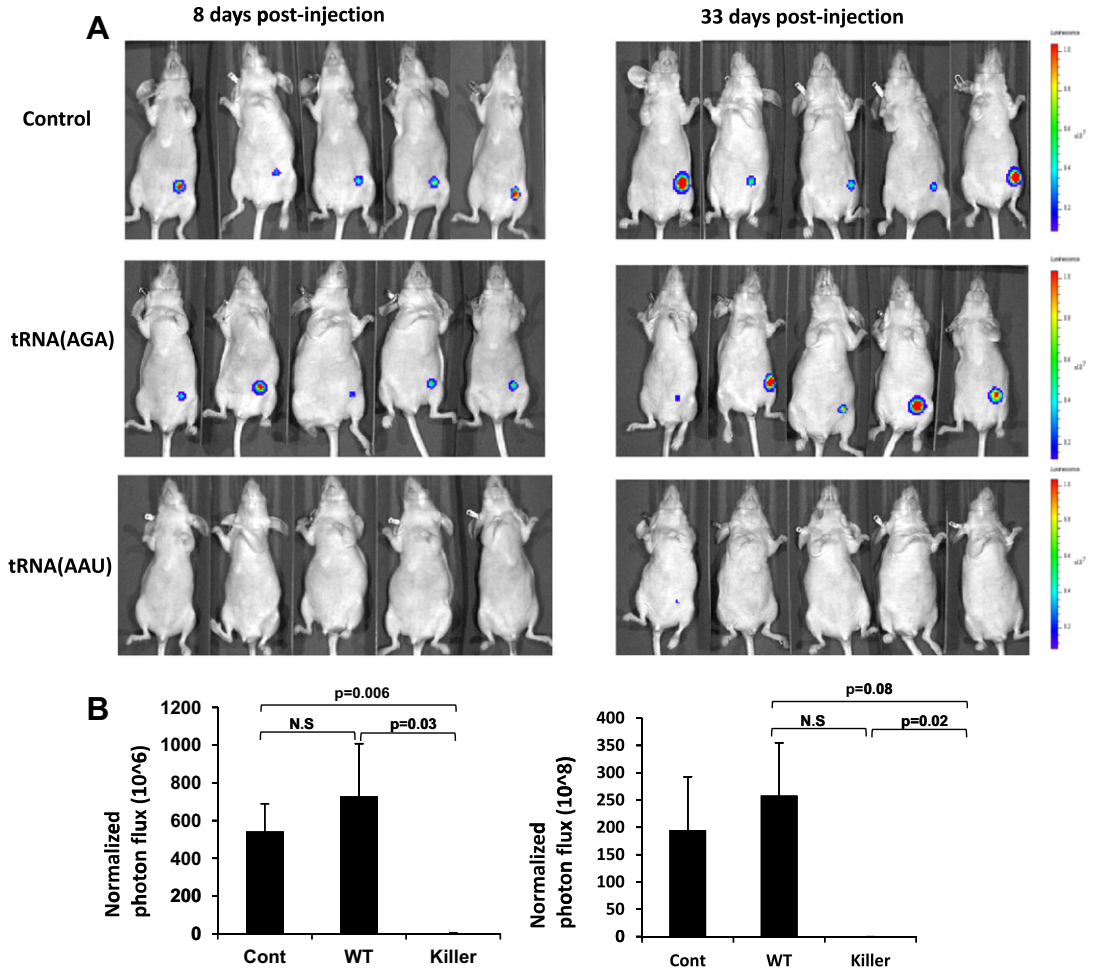

Fig. 4. tRNA^{Ser}(AAU) inhibits tumor growth in mice. (A) tRNA^{Ser}(AAU) inhibits potentiation of tumor growth in mice. 10^6 MDA-MB-231+luc cells were injected into each mouse at day 1. After 8 and 33 days, *in vivo* luciferase activity was imaged using the imaging system (IVIS) to monitor tumor growth and development. (B) The photon flux from the tumor is proportional to the number of light emitting cells and the size of the tumor.

cells are highly metastatic and have been used routinely in the field as a model system for xenograft tumors.

We first optimized the amount of tRNA^{Ser}(AAU) and the treatment time needed prior to mouse injection. Our previous experiments involved transfecting 5–75 ng tRNA^{Ser}(AAU) into each well of a 96-well plate containing 10^4 cells/well. However, 10^6 cells are needed for each mouse injection. To optimize the tRNA^{Ser}(AAU) level and transfection time, we initially used 6-well plates containing 3×10^5 cells per well and varied the dosage of tRNA^{Ser}(AAU) over a 24 h transfection period (Fig. 3A). We found that ~800 ng per well tRNA^{Ser}(AAU) represented the optimum concentration for transfection. Higher dosage led to greater side effects for even the wild-type tRNA^{Ser}(AGA), whereas lower dosage decreased the effect of tRNA^{Ser}(AAU). We then varied the length of transfection time at 800 ng tRNA^{Ser}(AAU) per well to determine whether tRNA^{Ser}(AAU) treatment can be reduced to less than 24 h (Fig. 3B). In this experiment, tRNA^{Ser}(AAU) was transfected for the indicated amount of time, both tRNA^{Ser}(AAU) and the transfection reagent were then removed from the medium, and the cells were incubated for another 24 h before analysis by the Wst-1 assay. We found that 8–16 h tRNA^{Ser}(AAU) treatment provides stable inhibition for MDA-MB-231+luc cells.

These conditions were used for tRNA^{Ser}(AAU) treatment and dosage during the actual mouse injection experiment. To obtain 10^6 cells for injection per mouse, we further scaled up tRNA^{Ser}(AAU) treatment. MDA-MB-231+luc cells in 10 cm plates at 80% confluence were transfected with 4000 ng tRNA^{Ser}(AAU), 4000 ng tRNA^{Ser}(AGA), or no-tRNA but containing the transfection reagent and incubated for 12 h. The transfection reagent and tRNA were removed, and cells were returned to normal medium for another 12 h to minimize the undesirable side effects induced by the transfection reagent. These three groups of cells were then harvested for mouse injection. A small aliquot of these same cells was also cultured to confirm that the tRNA^{Ser}(AAU) treatment indeed led to inhibition of cell growth (Fig. 3C).

Consistent with cell culture results, tRNA^{Ser}(AAU) had a large inhibitory effect on breast tumor formation in a xenograft mouse model (Fig. 4). We injected 10^6 cells into each mouse and monitored tumor formation after 8 and 33 days. As expected, 8/9 mice in the no-tRNA group developed tumors, and 7/10 mice in the tRNA^{Ser}(AGA) group developed tumors after 8 days. In contrast, no mice (0/10) in the tRNA^{Ser}(AAU) group developed tumors after 8 days. The same result was obtained at 33 days post-injection. These results demonstrate that tRNA^{Ser}(AAU) exhibits a strong inhibitory effect on tumor formation, and twelve hours of treatment are sufficient to achieve complete inhibition.

The work presented here demonstrates for the first time the ability of tRNA^{Ser}(AAU) to kill breast cancer cells. We also show that tRNA^{Ser}(AAU) can completely inhibit xenograph tumor formation in mice after treating a metastatic cell line for just 12 h. Recently, RNA molecules have been proposed as one of the next waves of therapeutic agents [16,17]. Most RNA-derived therapy

focuses on RNA interference where the primary mode of action is to target specific mRNAs to inhibit gene expression. In contrast, our tRNA-based approach does not target specific genes; rather, it tricks the ribosomes to produce mutant proteins that can misfold or aggregate. This proteome-wide, protein misfolding effect triggers intrinsic cellular stress response pathways, eventually leading to cell apoptosis. The most obvious advantage of this tRNA-based approach is that it may be very difficult for cancer cells to develop resistance to it. $tRNA^{Ser}$(AAU) is just like any other tRNA in cells, and ribosomes cannot mutate in such a way to avoid utilizing this tRNA.

The fundamental obstacle of tRNA-based therapy is the same as that of all other RNA-based therapies: namely, delivering this tRNA to specific cells of interest. Our results also suggest that such toxic, ''killer'' tRNAs preferentially kill tumor cells relative to normal epithelial breast cells and therefore have potential utility as selective anti-tumor agents. However, the possibility of toxicity in normal cells makes developing targeted delivery to tumorigenic cells a high priority in order to develop the therapeutic potential of tRNAs such as $tRNA^{Ser}$(AAU).

Acknowledgments

We thank Eva Eves for helpful comments. This work was supported by a grant from CDMRP (W81XWH-10-1-0104, 10-1-0105 to TP and MRS) and from NIH (T32HL094282 to CF).

References

[1] T. O'Hare, C.A. Eide, M.W. Deininger, Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia, Blood 110 (2007) 2242–2249.
[2] C.H. Yun, K.E. Mengwasser, A.V. Toms, M.S. Woo, H. Greulich, K.K. Wong, M. Meyerson, M.J. Eck, The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP, Proc. Natl. Acad. Sci. USA 105 (2008) 2070–2075.
[3] F. Leonessa, R. Clarke, ATP binding cassette transporters and drug resistance in breast cancer, Endocr. Relat. Cancer 10 (2003) 43–73.
[4] D.B. Longley, P.G. Johnston, Molecular mechanisms of drug resistance, J. Pathol. 205 (2005) 275–292.
[5] P.I. Poulikakos, C. Zhang, G. Bollag, K.M. Shokat, N. Rosen, RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF, Nature 464 (2010) 427–430.
[6] Y. Kozutsumi, M. Segal, K. Normington, M.J. Gething, J. Sambrook, The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins, Nature 332 (1988) 462–464.
[7] R.C. Wek, H.Y. Jiang, T.G. Anthony, Coping with stress: eIF2 kinases and translational control, Biochem. Soc. Trans. 34 (2006) 7–11.
[8] S.H. Back, R.J. Kaufman, Endoplasmic reticulum stress and type 2 diabetes, Annu. Rev. Biochem. 81 (2012) 767–793.
[9] C. Xu, B. Bailly-Maitre, J.C. Reed, Endoplasmic reticulum stress: cell life and death decisions, J. Clin. Invest. 115 (2005) 2656–2664.
[10] R. Geslain, L. Cubells, T. Bori-Sanz, R. Alvarez-Medina, D. Rossell, E. Marti, L. Ribas de Pouplana, Chimeric tRNAs as tools to induce proteome damage and identify components of stress responses, Nucleic Acids Res. 38 (2009) e30.
[11] S. Cusack, Aminoacyl-tRNA synthetases, Curr. Opin. Struct. Biol. 7 (1997) 881–889.
[12] R. Geslain, T. Pan, Functional analysis of human tRNA isodecoders, J. Mol. Biol. 396 (2010) 821–831.
[13] J.F. Milligan, O.C. Uhlenbeck, Synthesis of small RNAs using T7 RNA polymerase, Methods Enzymol. 180 (1989) 51–62.
[14] M. Pavon-Eternod, S. Gomes, R. Geslain, Q. Dai, M.R. Rosner, T. Pan, TRNA overexpression in breast cancer and functional consequences, Nucleic Acids Res. 37 (2009) 7268–7280.
[15] S. Dangi-Garimella, J. Yun, E.M. Eves, M. Newman, S.J. Erkeland, S.M. Hammond, A.J. Minn, M.R. Rosner, Raf kinase inhibitory protein suppresses a metastasis signalling cascade involving LIN28 and let-7, EMBO J. 28 (2009) 347–358.
[16] N.M. Snead, J.J. Rossi, RNA interference trigger variants: getting the most out of RNA for RNA interference-based therapeutics, Nucleic Acid Ther. 22 (2012) 139–146.
[17] J.C. Burnett, J.J. Rossi, RNA-based therapeutics: current progress and future prospects, Chem. Biol. 19 (2012) 60–71.